The Cholera Toxin B Subunit is a Mucosal Adjuvant for Oral Tolerance Induction in Type 1 Diabetes

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Abstract

When conjugated to various proteins, the nontoxic B-chain of cholera toxin (CTB) significantly increases the ability of these proteins to induce immunological tolerance after oral administration. Here, we investigated if a nonconjugated form of CTB enhances the induction of immune tolerance after oral insulin administration. Induction of immunological tolerance was studied after oral administration of insulin preparations in three mouse models; an insulin/ovalbumin coimmunization model, a model of virus-induced diabetes in transgenic RIP-LCMV-NP mice and in nonobese diabetic (NOD) mice serving as a model of spontaneous diabetes. In the immunization model, we demonstrate that mixing with CTB increases the tolerogenic potential of insulin, approximately 10 fold. Titration of the CTB concentration in this system revealed that an insulin : CTB ratio of 100 : 1 was optimal for the induction of bystander suppression. Further studies revealed that this insulin : CTB ratio also was optimal for the prevention of diabetes in a virus-induced, transgenic diabetes model. In addition, the administration of this optimal insulin–CTB preparation significantly prevented the onset of diabetes in old NOD mice with established islet infiltration. The data presented here demonstrate that CTB, even in its unconjugated form, functions as a mucosal adjuvant, increasing the specific tolerogenic effect of oral insulin.

Introduction

The cause for insulin-dependent type 1 diabetes resulting from immune-mediated destruction of the insulin-secreting β-cells in the pancreas remains elusive [1, 2]. Based on studies in nonobese diabetic (NOD) mice, several hypotheses focus on dysregulation of the immune system as an initiating factor in the pathogenesis. Hence, the low peptide-binding affinity of the unique major histocompatibility complex class II molecule that in part confers the genetic susceptibility to diabetes in NOD mice, I-A^{g7} [3], could eventually lead to the absence of antidiabetogenic, regulatory T cells. In conjunction with still unknown genetic or environmental factors, this absence of regulatory T cells is believed to facilitate the infiltration of the pancreas by macrophages and dendritic cells, 3–4 weeks after birth. This is followed by a period of progressive T-cell-mediated destruction of the insulin-producing β-cells, starting from 10–20 weeks of age and eventually resulting in clinical diabetes manifestation. This autoaggressive process is driven by T helper 1 (Th1)-like $CD4^+$ and cytotoxic $CD8^+$ T cells reactive to β-cell autoantigens such as insulin, GAD65, IAC512 and hsp60 [1, 2, 4]. Thus, one possible intervention point in the pathogenesis of autoimmune diabetes would be to induce immune-regulatory, antidiabetogenic T cells able to eliminate or downregulate the autoaggressive T cells, causing the autoimmune destruction of pancreatic β-cells [5].

Peripheral tolerance or immune modulation rendering mature lymphocytes hyporesponsive to dietary antigens (Ags) or 'bystander-suppressing' responses to other unrelated antigens is termed oral tolerance [6]. Oral tolerance induced by autoantigens has recently been applied successfully as a therapeutic tool in experimental models of autoimmune diseases [7, 8]. Although the basic mechanisms of oral tolerance are operational in humans [9], oral Ag-administration regimens have showed limited success when applied to patients [8, 10–12]. One possible explanation for this could be the fact that the doses of orally administrated antigens used in humans were relatively

low compared with those used in rodents [13], especially while taking the surface area of the intestinal absorptive epithelium into consideration. Alternatively, CTB might provide efficient costimulation needed to overcome an inefficient presentation of insulin to the mucosal T cells, resulting from the limited transport of native insulin over the epithelial barrier. Either way, to be able to treat human autoimmune diseases with orally administrated Ags, very high doses would be necessary [13], thereby rendering these regimens infeasible. Thus, for oral tolerance to become a realistic therapeutic regimen in human autoimmune diseases, adjuvants with the ability to enhance the tolerogenic potential of ingested Ags need to be identified. Coupling autoantigens to the nontoxic B-chain of cholera toxin, CTB, dramatically increases their tolerogenic potential after oral administration [14–20]. This effect is probably mediated by the ability of CTB to act as a mucosal carrier system [15], although CTB might also have direct effects on the immune system [21, 22]. Despite being a potential candidate for a mucosal carrier protein, the complicated insulin–CTB conjugation process [17] might prevent its use in industrial scale production of an oral diabetes vaccine. Thus, in the present paper, we have investigated whether CTB in a nonconjugated form can act as a mucosal adjuvant, augmenting the ability of insulin to downregulate an autoaggressive response, and whether such mixtures of insulin and CTB can prevent the development of both virus-induced and spontaneous autoimmune diabetes.

Materials and Methods

Mice. Female BALB/*c* and NOD mice were purchased at M&B (Ry, Denmark) and housed in the central animal facility at Novo Nordisk (Bagsværd, Denmark). Mice expressing the nuclear protein (NP) of lymphocytic choriomeningitis virus (LCMV) under the rat insulin promoter (RIP-LCMV-NP H-2^d mice) have been previously described [2] and were housed in the animal facility at the Scripps Research Institute (La Jolla, CA, USA).

Reagents and chemicals. Human insulin was used as pure crystals obtained form the last step of the production at Novo Nordisk, immediately before use in drug formulation. For oral administration, insulin was diluted in 0.35 M $NaHCO_3$ (Merck, Darmstadt, Germany). Ovalbumin (OVA, grade V), concanavalin A (Con A), recombinant monosialoganglioside (rGM-1) and complete Freund's adjuvant (CFA) were purchased at Sigma Chemicals (St. Louis, MO, USA). Recombinant CTB was supplied by SBL (Uppsala, Sweden).

Immunization model. Female BALB/*c* mice were treated p.o. with vehicle, CTB, OVA and with the indicated insulin–CTB formulations five times per week for a total of 4 weeks. After 2 weeks of treatment, mice were immunized with both 50 μg of insulin and 100 μg of OVA in CFA in the footpad, as previously described [23]. Two weeks later, mice were bled and sacrificed, and the popliteal lymph nodes (pLNs) were excised and pooled for each group. Single-cell suspensions were generated by passage of pLNs through a nylon filter. Quadruplicate cultures of 2×10^5 cells were established in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in a total volume of 200 μl in the presence of phosphate-buffered saline, 100 μg/ml of OVA or 1 mg/ml of insulin. The cultures were incubated for 72 h in a humid 5% CO_2 atmosphere. For the last 6 h of the incubation period, 0.5 μCi ^{3}H-TdR (Amersham, Little Chalfont, UK) was added to the plates. The procedures for harvesting the plates and measurement of the incorporated radioactivity have previously been described [24].

In separate experiments, the supernatants were harvested from day 3 *in vitro* cultures, and the amounts of interleukin-4 (IL-4) and interferon-γ (IFN-γ) were measured using commercial enzyme-linked immunosorbent assay kits, following the manufacturer's instructions (Pharmingen, San Diego, CA, USA).

LCMV-induced diabetes. The purification of LCMV and the induction of diabetes in RIP-LCMV mice have previously been described in detail [2, 25]. In brief, RIP-LCMV mice were treated with vehicle, 1 μg of CTB, 1 mg of insulin, 100 μg insulin + 100 μg CTB, 100 μg insulin + 10 μg CTB, 100 μg insulin + 1 μg CTB or 100 μg insulin + 10 μg CTB + 10 μg rGM-1 in 0.25 ml twice per week, starting 1 week prior to i.p. infection with 10^5 p.f.u. of LCMV Armstrong strain. Mice were screened for hyperglycaemia by weekly tail vein bleedings, followed by measurements of blood glucose levels using a Glucometer (Bayer, Leverkusen, Germany). Mice with a blood glucose level above 15 mM were scored as diabetic.

Diabetes in NOD mice. Female NOD mice were screened for diabetes weekly, until 22 weeks of age. All mice developing hyperglycaemia (<10 mM) in this period, 40–45% of the mice, were excluded from the study. Non-diabetic 22-week-old female NOD mice were treated p.o. with vehicle, CTB, insulin or insulin–CTB admixtures in 0.25 ml, five times per week for at total of 10 weeks. Mice were screened for hyperglycaemia as described above.

Results

Admixtures of insulin and CTB induce bystander suppression after oral administration

We wanted to investigate whether CTB is a mucosal adjuvant capable of increasing the tolerogenic potential of insulin after oral administration. In initial experiments, we used an immunization model where mice were fed with insulin and immunized in the footpad with OVA and insulin. In this model, oral administration of insulin inhibits the *in vitro* T-cell response to OVA when mice are coimmunized with the two antigens but not when mice are

immunized with insulin and OVA at distinct sites, pinpointing the bystander nature of this suppression (Fig. 1A) [23]. Figure 1B shows that in this system, oral administration of 1 mg of insulin mediated an approximately 50% suppression of the *ex vivo* OVA-specific proliferation, whereas 100 μg of insulin had no effect. However, when 100 μg of insulin was admixed 1 : 1 with CTB, a 35% suppression of the *in vitro* response to OVA was observed (Fig. 1B). This suppressive effect of insulin was increased further when the CTB concentration was lowered, consistently reaching >90% suppression when 100 μg of insulin was admixed with 1 μg of CTB (Fig. 1B). However, when decreasing the CTB concentration yet 10 fold, the adjuvant effect of CTB was lost. The tolerogenic effect of CTB was not because of the deletion of pLN T cells, as no differences in cell numbers and Con A-induced proliferation were observed between the groups (Table 1). In addition, the OVA-specific production of both IL-4 and IFN-γ was almost completely suppressed in mice treated with either 1000 μg of insulin or 100 μg of insulin admixed with 1 μg of CTB (Table 2). These findings demonstrate that CTB acts as mucosal adjuvant, increasing the ability of insulin to induce bystander suppression of both Th1 and Th2 T cells. Attempts to record insulin-specific *in vitro* proliferation were all negative, probably because of the very low antigenicity of insulin (data not shown).

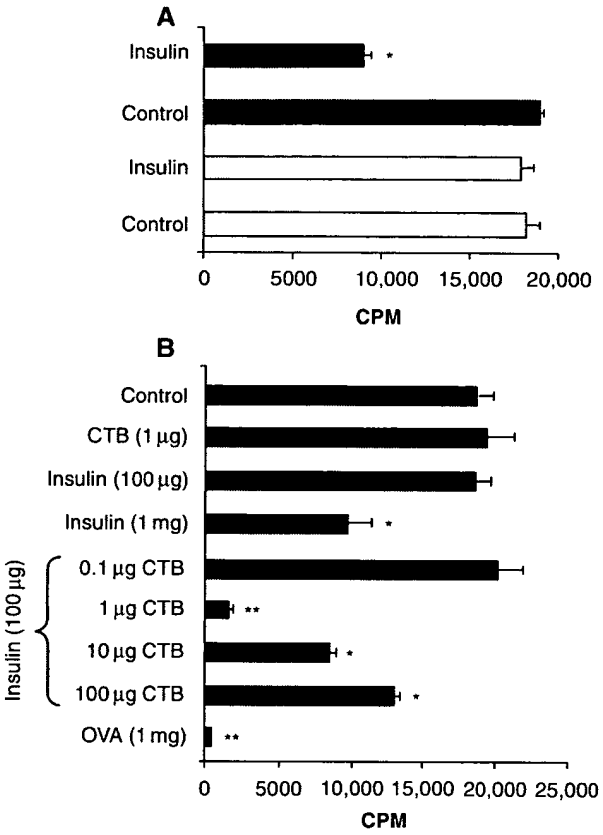

Figure 1 Insulin–cholera toxin B-chain (CTB) admixtures induce bystander suppression *in vivo*. (A) Female BALB/*c* mice were fed daily five times per week for 4 weeks with vehicle or 1 mg of insulin by intragastric gavage. After 2 weeks of feeding, mice were either immunized in the footpad with insulin and ovalbumin (OVA) in complete Freund's adjuvant (CFA) (black bars) or immunized with OVA in the footpad and with insulin subcutaneously in the neck, both in CFA (open bars). Mice were sacrificed after an additional 2 weeks, and the OVA-specific proliferation of popliteal lymph node (pLN)-derived T cells was assessed *in vitro*. (B) Female BALB/*c* mice were fed daily five times per week for 4 weeks with vehicle, CTB, insulin or with the indicated admixtures of insulin and CTB by intragastric gavage. After 2 weeks of feeding, mice were immunized in the footpad with insulin and OVA in CFA. Mice were sacrificed after an additional 2 weeks, and the OVA-specific proliferation of pLN-derived T cells was assessed *in vitro*. Data represent the mean from quadruplicate cultures, and standard deviation values are indicated. Asterisks denote statistical significance from vehicle-treated mice, $^{*}P < 0.01$, $^{**}P < 0.005$.

Admixtures of insulin and CTB prevent diabetes in RIP-LCMV-NP mice

Next, we wanted to investigate whether the bystander suppression induced by oral administration of insulin–CTB admixtures could prevent the development of autoimmune diabetes. For these experiments, we used mice transgenic for the NP of LCMV under the rat insulin promoter (RIP-LCMV) [2]. When infected with LCMV, these mice clear the virus infection but develop diabetes within 3 weeks after infection owing to immune-mediated destruction of the LCMV-NP-expressing β-cells [2, 25]. RIP-LCMV mice were treated with vehicle and various formulations of insulin twice per week, starting 1 week prior to virus infection. Figure 2A shows that 4 weeks after virus infection, 90% of the mice treated with vehicle had developed diabetes. When mice were fed with 1 mg of human insulin or 100 μg of insulin admixed 1 : 1 with CTB, only a marginal protection was observed (Fig. 2B,C). However, when mice were fed with 100 μg of human insulin admixed with either 10 μg or 1 μg of CTB, a 50–60% reduction in the diabetes incidence was observed ($P < 0.02$ and $P < 0.05$, respectively) (Fig. 2D,E). No significant differences in the levels of hyperglycaemia in the mice developing diabetes were observed between the groups (data not shown). Feeding of mice with 1 μg of CTB had no effect on the incidence of diabetes, thus excluding a direct CTB-mediated effect (data not shown). When rGM-1 was included in the admixtures, the protective effect of the insulin treatment was abrogated, demonstrating that the adjuvant effect was indeed mediated by CTB binding to its receptor rGM-1 (Fig. 2F). Together, these show that insulin–CTB admixtures can induce active immune suppression to irrelevant Ags, expressed in the same micromilieu as insulin.

Insulin–CTB admixtures prevent spontaneous diabetes in NOD mice

To investigate whether insulin–CTB admixtures would be effective in preventing spontaneous autoimmune diabetes,

Table 1 Cell number and concanavalin A (Con A) response of popliteal lymph node (pLN)-derived cells

Oral treatment*				
Insulin (μg)	CTB (μg)	OVA (μg)	Cell number ($\times 10^6$)†	CPM‡
–	–	–	3.21 ± 0.51§	27,359 ± 2351¶
–	1	–	3.62 ± 0.41	29,801 ± 3837
1000	–	–	3.07 ± 0.58	35,436 ± 7554
100	–	–	2.89 ± 0.61	31,686 ± 3852
100	0.1	–	3.37 ± 0.33	29,257 ± 4930
100	1	–	3.42 ± 0.42	26,127 ± 4453
100	10	–	3.27 ± 0.39	28,912 ± 1021
100	100	–	2.98 ± 0.62	33,465 ± 5709
–	–	1000	3.14 ± 0.44	30,993 ± 4022

Similar results were obtained in a repeat experiment. CTB, cholera toxin B-chain; OVA, ovalbumin.
*Mice were treated daily as indicated for at total of 4 weeks, after 2 weeks mice were immunized in the foot pad with OVA and insulin in complete Freund's adjuvant (CFA). After additional 2 weeks, the mice were sacrificed and pLNs dissected.
†pLNs were harvested from mice treated as indicated and leuckocytes were isolated.
‡Con A-induced proliferation (experimental counts per minute (CPM) – background CPM). Background CPM were always less than 5% of the experimental values.
§Data represent the mean from groups of five mice ± standard error of mean.
¶Data represent the mean from quadruplicate cultures ± standard deviation.

we chose to treat old NOD mice with insulin–CTB admixtures as these have already established insulitis, and thus more closely resemble type 1 patients presented in the clinic. Thus, 22 week nondiabetic NOD mice were treated daily with vehicle, insulin, CTB or insulin–CTB admixtures for 10 weeks. Figure 3A–C shows that 60% of the vehicle-treated mice and 50% of the insulin- and the CTB-treated, respectively, developed diabetes within the treatment period. In contrast, only 20% of the mice treated with the insulin–CTB admixture developed diabetes within this period ($P < 0.01$) (Fig. 3D). These data demonstrate that insulin–CTB admixtures are able to halt the diabetogenic process in mice with already established insulitis, underscoring the potential of this treatment principle in human diabetes.

Discussion

We found that in its nonconjugated form, CTB can function as a mucosal adjuvant, increasing the potency of human insulin to induce bystander suppression *in vivo* after oral administration. Furthermore, we show that admixtures of insulin and CTB prevent diabetes in a virus-induced transgenic mouse model as well as in spontaneous diabetic NOD mice. Together, these data identify CTB–insulin admixtures as a possible therapeutic regimen in autoimmune diabetes.

Coupling of autoantigens to CTB has previously been demonstrated to increase their tolerogenic potential after oral administration by up to 100 fold [14–20]. This effect has mainly been attributed to the ability of CTB to act as a mucosal carrier protein, securing efficient translocation of the coupled antigens from the intestinal lumen over the absorptive epithelium via the GM-1 receptor [15]. The effect of mixing insulin with CTB demonstrated here is probably not mediated by such a carrier effect, as it seems less likely that CTB should facilitate the cotransfer of insulin over the epithelium via GM-1. It is more likely that CTB acts as an adjuvant that could modulate the function of antigen-presenting cells by increasing their costimulatory potential [26], leading to increased generation of regulatory T cells in the Th2 cytokine milieu

Table 2 Ovalbumin(OVA)-induced interleukin-4 (IL-4) and interferon-γ (IFN-γ) production *in vitro*

Oral treatment*		Cytokine production	
Insulin (μg)	CTB (μg)	IL-4 (pg/ml)	IFN-γ (pg/ml)
–	–	725 ± 122†	3014 ± 347†
1000	–	147 ± 154 ($P < 0.01$)	478 ± 266 ($P < 0.005$)
100	–	692 ± 165	3351 ± 703
–	1	809 ± 200	2999 ± 289
100	1	132 ± 117 ($P < 0.01$)	403 ± 311 ($P < 0.005$)

Similar results were obtained in a repeat experiment. CTB, cholera toxin B-chain.
*Mice were treated daily as indicated for at total of 4 weeks. After 2 weeks, mice were immunized in the foot pad with OVA and insulin in complete Freund's adjuvant (CFA). After additional 2 weeks, the mice were sacrificed and popliteal lymph nodes dissected.
†Data represent the mean from quadruplicate cultures ± standard deviation.

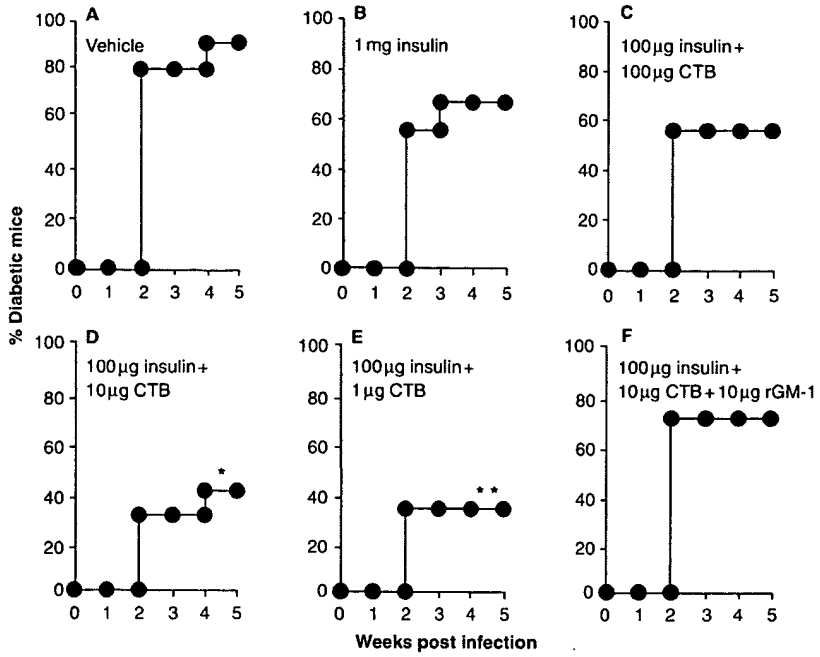

Figure 2 Insulin–cholera toxin B-chain (CTB) admixtures prevent diabetes in RIP-LCMV mice. Female RIP-LCMV mice were treated with (A) vehicle, (B) 1 mg of insulin, (C) 100 µg insulin + 100 µg CTB (D) 100 µg insulin + 10 µg CTB, (E) 100 µg insulin + 1 µg CTB or (F) 100 µg insulin + 10 µg CTB + 10 µg recombinant monosialoganglioside (rGM-1), 1 week prior to i.p. infection with 10^5 p.f.u. of LCMV Armstrong strain. Mice were screened for hyperglycaemia weekly. Data are from nine to 10 mice per group. Asterisks denote statistical significant differences in average diabetes-free period from vehicle-treated mice, $^*P \leq 0.05$, $^{**}P \leq 0.02$.

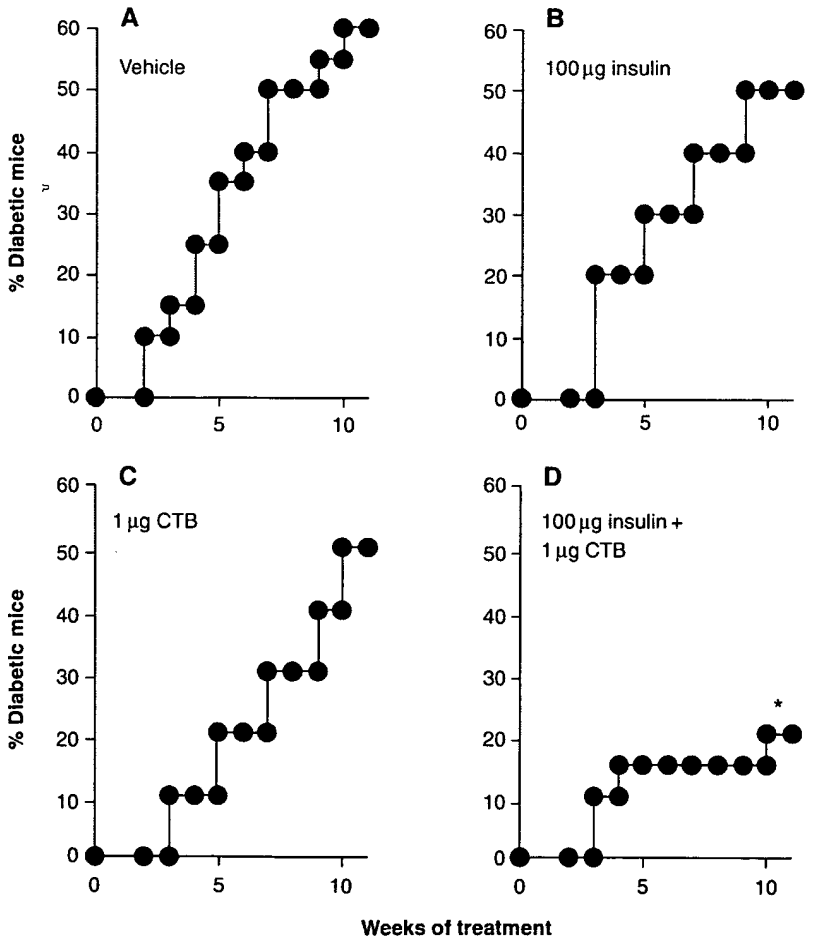

Figure 3 Insulin–cholera toxin B-chain (CTB) admixtures prevent the onset of diabetes in old nonobese diabetic (NOD) mice. Twenty-two-week-old female NOD mice were treated orally five times per week with (A) vehicle, (B) 100 µg of insulin, (C) 1 µg of CTB or (D) 100 µg insulin + 1 µg CTB. Mice were screened for hyperglycaemia weekly. Data are from groups of 20 mice. Asterisks denote statistical significant differences in average diabetes-free period from vehicle-treated mice, $^*P \leq 0.01$.

existing in the intestinal mucosa [6]. The ability of CTB to downmodulate Th1-like macrophages as reported by Burkart *et al.* [21] might further facilitate the induction of an immune-regulatory T-cell response.

Two mechanisms are believed to account for the induction of oral tolerance. Single administration of high Ag doses induces clonal deletion of Ag-specific lymphocytes. In contrast, multiple low doses of Ags induce tolerance by inducing regulatory T cells, with the potential to actively downregulate peripheral immune responses [6]. Oral tolerance induced by insulin–CTB admixtures is most likely because of the induction of active immune-regulatory cells, as the admixtures mediated the downmodulation of the response to an irrelevant Ag as OVA when these are expressed in the same tissue, thereby underscoring the bystander nature of this suppression [23, 25, 27, 28]. Moreover, no inhibition of insulin-specific antibody response was seen in these experiments (data not shown) – what would have been expected, if the admixtures were inducing clonal deletion, as B cells are more prone to clonal deletion than T cells [6].

Oral administration of insulin and insulin–CTB conjugates has been demonstrated to induce $CD4^+$ regulatory T cells that are central in the prevention of disease [25, 27, 28]. As described above, insulin–CTB admixtures induce bystander suppression *in vivo*. This ability of insulin–CTB admixtures is further underscored by their ability to block the development of virus-induced diabetes in the RIP-LCMV mice. This model allows the dissection of the disease-inducing Ags from the tolerance-inducing Ags, as the autoimmune process is driven by an LCMV epitope expressed by β-cells in these mice. T-cell reactivity to several islet autoantigens has been identified in autoimmune diabetes [1, 4]. Therefore, the ability to induce the suppression of bystander reactivity is highly significant in diabetes prevention, as it confers CTB–insulin-induced regulatory T cells the ability to suppress the function of pathogenic, autoaggressive T cells, irrespective of their Ag specificity. Treatment with insulin–CTB admixtures starting at 22 weeks of age significantly protects NOD mice from diabetes, indicating that this regimen can halt the further deterioration of already established insulitis [1, 3]. Previously, oral administration of insulin and insulin–CTB conjugates has been demonstrated to induce $CD4^+$ regulatory T cells that are central in the prevention of disease [25, 27, 28]. Induction of $CD4^+CD25^+$ regulatory T cell has been reported in other experimental models of oral tolerance [29, 30]. $CD4^+CD25^+$ regulatory T cells are believed to mature in the intestinal mucosa upon exposure to foreign Ags in the presence of IL-4 [31–33]. Mature $CD4^+CD25^+$ regulatory T cells display a Th3 phenotype, secreting high amounts of tumour growth factor-β when exposed to relevant antigens [32, 33]. The immune suppressive potential of $CD4^+CD25^+$ regulatory T cells has been demonstrated in a number of settings [32, 33]. Moreover, it has been demonstrated that NOD mice have reduced numbers of $CD4^+CD25^+$ regulatory T cells [34], and that these cells can prevent onset of diabetes in NOD mice [34, 35]. Thus, although not formally demonstrated, it is likely that $CD4^+CD25^+$ regulatory T cells mediate the suppression of autoaggressive T cell after oral administration of insulin–CTB mixtures.

The adjuvant effect of CTB appears inversely correlated with dose. Hence, 1 μg was more effective than 10 and 100 μg. A probable explanation could be that high concentrations of CTB induce apoptotic cell death in activated T cells, as recently demonstrated in an experimental model of colitis [36] and in various *in vitro* systems [37, 38]. However, in the RIP-LCMV mice 1 and 10 μg are equally effective. This might be explained by the less frequent administration of insulin–CTB mixtures in these mice, only resulting in a sufficient CTB exposure for the induction of T-cell apoptosis in the mice exposed to 100 μg of CTB.

It could be speculated that diabetes prevention could be owing to a metabolic effect of the oral insulin via increased β-cell rest and survival. A minimal metabolic effect of oral insulin was previously reported [39]; however, it seems unlikely that the prevention reported here was because of a metabolic effect of oral insulin as 1 mg of human insulin had only a negligible effect on the incidence of virus-induced diabetes. Likewise, 100 μg of insulin did not affect the onset of diabetes in NOD mice, and only became protective when admixed with CTB. Thus, it seems most likely that the protective effect observed here is owing to peripheral immunological tolerance induced by the CTB–insulin admixtures.

In conclusion, we have demonstrated that CTB is an effective mucosal adjuvant, with the ability to increase the tolerogenic potential of orally administered insulin. In addition to its relevance for the treatment of type 1 diabetes, the use of CTB as an adjuvant might prove to be an important tool in the development of new immune-regulatory treatment regimens in other autoimmune disorders.

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